

## Research Article

Detecting and characterizing the highly divergent plastid genome of the nonphotosynthetic parasitic plant *Hydnora visseri* (Hydnoraceae)

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## Abstract

Plastid genomes of photosynthetic flowering plants are usually highly conserved in both structure and gene content. However, the plastomes of parasitic and mycoheterotrophic plants may be released from selective constraint due to the reduction or loss of photosynthetic ability. Here we present the greatly reduced and highly divergent, yet functional, plastome of the nonphotosynthetic holoparasite *Hydnora visseri* (Hydnoraceae, Piperales). The plastome is 27 kb in length, with 24 genes encoding ribosomal proteins, ribosomal RNAs, tRNAs and a few non-bioenergetic genes, but no genes related to photosynthesis. The inverted repeat and the small single copy region are only ~1.5 kb, and intergenic regions have been drastically reduced. Despite extreme reduction, gene order and orientation are highly similar to the plastome of *Piper cenocladum*, a related photosynthetic plant in Piperales. Gene sequences in *Hydnora* are highly divergent and several complementary approaches using the highest possible sensitivity were required for identification and annotation of this plastome. Active transcription is detected for all of the protein coding genes in the plastid genome, and one of two introns is appropriately spliced out of *rps12* transcripts. The whole genome shotgun read depth is 1,400X coverage for the plastome, while the mitochondrial genome is covered at 40X and the nuclear genome at 2X. Despite the extreme reduction of the genome and high sequence divergence, the presence of syntenic, long transcriptionally-active open reading frames with distant similarity to other plastid genomes and a high plastome stoichiometry relative to the mitochondrial and nuclear genomes suggests that the plastome remains functional in *Hydnora visseri*. A four stage model of gene reduction, including the potential for complete plastome loss, is proposed to account for the range of plastid genomes in nonphotosynthetic plants.

Key words: Parasitic Plants, holoparasite, nonphotosynthetic, Hydnoraceae, Plastome, Plastid Genome

## Introduction

The plastids of green plants (Viridiplantae) are double membrane-bound organelles derived from cyanobacteria via endosymbiosis. The primary function of plastids in most green algae and land plants is the fixation of carbon dioxide via photosynthesis (chloroplasts), however plastids may also function in storage of starch (amyloplasts), lipids (elaioplasts), or proteins (proteinoplasts) (Bock, 2007; Wicke et al. 2011; Ruhlman and Jansen, 2014). Plastids maintain a separate, circular-mapping, DNA genome that is uniparentally inherited independent of the nuclear and mitochondrial genomes. The plastid genome (plastome) of land plants is usually about 120-170 kb in size, is highly conserved in photosynthetic plants, and typically encodes about 120-130 genes (reviewed in Ruhlman and Jansen 2014). Plastome organization is highly conserved, containing a large single copy region (LSC) and a small single copy region (SSC) separated by two copies of an inverted repeat (IRa, IRb) (Shinozaki et al. 1986; Ohyama et al. 1986; Jansen et al. 2007).

Parasitic and mycoheterotrophic plants have repeatedly reduced or lost the need for photosynthesis in their chloroplasts by establishing a physiological connection with host plants or fungi to obtain carbohydrates, water, and other nutrients. The reduced demand for photosynthetic ability in these heterotrophic plants has relaxed or eliminated evolutionary constraints on photosynthesis and other genes related to plastid function, resulting in a divergent and greatly reduced plastid genome (dePamphilis and Palmer 1990; Wicke et al. 2013; Barrett et al. 2014). The first plastid genome of a nonphotosynthetic plant was sequenced over twenty years ago (*Epifagus virginiana*, Wolfe et al. 1992).

Currently, there are over 535 complete plastid genomes of land plants deposited in GenBank (Retrieved 1 June 2015). Among the published parasitic flowering plant plastomes, twelve are members of the broomrape family (Orobanchaceae) (Wolfe et al. 1992; Wicke et al. 2013, Li

et al. 2013; Uribe-Convers et al. 2014), four are dodders (*Cuscuta*) in the morning glory family (Convolvulaceae) (McNeal et al. 2007; Funke et al. 2007), and four are mistletoes from the Santalales (Petersen et al. 2015). In addition, 17 plastomes have been sequenced for mycoheterotrophic plants, including several orchids (*Rhizanthella gardneri*, *Neottia nidus-avis*, *Epipogium aphyllum*, *Epipogium roseum*, and ten *Corallorhiza* species) (Delannoy et al. 2011; Logacheva et al. 2011; Schelkunov et al. 2015; Barrett and Davis 2012; Barrett et al. 2014), other monocots *Petrosavia stellaris* and *Sciaphila densiflora* (Logacheva et al. 2014; Lam et al. 2015), and the liverwort *Aneura mirabilis* (Wickett et al. 2008). All of these plastomes retain a core set of genes that support production of plastid ribosomes and one or several genes whose transcripts encode proteins that may be essential to plastid processes in nonphotosynthetic plants, including intron processing (*matK*), lipid synthesis (*accD*), and protein synthesis and processing. In nonphotosynthetic species, genes related to photosynthesis, transcription, and NAD(P)H dehydrogenase subunits are often nonfunctional or lost (Wicke et al. 2011; Barrett et al. 2014).

While the reduction in plastome gene content of nonphotosynthetic plants has been well documented, there has been a long-standing debate about the minimal plastid genome in the absence of photosynthetic constraint, and whether plastids or their genomes could be entirely lost in some heterotrophic plants (dePamphilis and Palmer 1990; Wolfe et al 1992; Nickrent et al. 1997; Bungard et al. 2004; Barbrook et al. 2006; Krause 2008; Wicke et al. 2011; Molina et al. 2014; Race et al. 1999; Schelkunov et al. 2015). Among nonphotosynthetic plant plastomes sequenced to date, 27 to 35 genes are typically retained (Wicke et al. 2011, Wicke et al. 2013, Barrett et al. 2014, Schelkunov et al. 2015, Lam et al. 2015), including machinery for translation (rRNA and ribosomal protein genes) that may be required only for the expression of a small number of potentially indispensable protein coding sequences (Wolfe et al. 1992; Krause and Scharff 2014 and references therein). While each of these core non-



bioenergetic, non-translational genes, including *accD*, *clpP*, *ycf1*, and *ycf2*, have been lost from the plastid genome in at least some photosynthetic angiosperm lineages (Wicke et al. 2011), these genes are typically retained in most nonphotosynthetic plants (Braukmann and Stefanović 2012, Barrett et al. 2014). Given enough evolutionary time, the ongoing process of gene transfer from the plastome to the nuclear and mitochondrial genomes could result in the functional transfer of the last of these essential non-bioenergetic, non-translational genes, and at that point, genes involved only in translational function would be unnecessary, allowing the continued deletion and potential complete loss of the plastome. Alternatively, some core sequences may not be transferrable out of the plastome because of redox balance requirements (Race et al. 1999) or other still unknown processes that require certain genes, or even nongenic sequences, to remain plastid encoded. The plastid *trnE* gene has been discussed as a compelling candidate out of all the plastid genes that has to be retained due to a dual function (Barbrook et al. 2006). Ancient holoparasitic lineages provide evolutionary test cases for the minimal plastid genome, and whether complete loss of the plastid genome has ever occurred.

In one recent study, the plastid genome appears to be missing from whole genome shotgun data from the holoparasitic flowering plant *Rafflesia lagascae* (Molina et al. 2014). Different search strategies failed to identify a plastid genome in the genomic assembly. Reference-based mapping, a BLASTn approach, and profile Hidden Markov Models of plastid gene alignments identified only short and low coverage fragments of plastid genes at less than 2X depth of coverage, whereas assembled portions of the mitochondrial genome were readily detected at much higher depth of coverage (350X). At the same time, another study reported the putative loss of the plastid genome from the nonphotosynthetic unicellular alga *Polytomella* (Smith and Lee 2014). Using related chlorophyte organellar genomes as queries, both BLAST and reference mapping based approaches from whole genomic Illumina data of

four *Polytomella* species did not recover any reads corresponding to the plastid genome. Compared to *Rafflesia*, where microscopy shows only a plastid-like organelle without a known function, *Polytomella* possesses starch-storing plastids (Moore et al. 1970; Brown et al. 1976).

In photosynthetic plants, there are typically dozens of copies of mitochondrial DNA and hundreds to thousands of copies of plastid DNA per cell. Therefore, in a genomic sequence sample where no attempt has been made to enrich for DNA from one genomic compartment or another, a plastid genome would be expected to have a relative depth of read coverage that is one or even two orders of magnitude greater than the mitochondrial genome and three to four orders of magnitude greater than the nuclear genome (Straub et al. 2011a; Straub et al. 2011b, Wolf et al. 2015). In a nonphotosynthetic plant, however, where the plastome has lost part of its functionality, the normal stoichiometric relationships may be altered. Nevertheless, stoichiometry is important evidence for the detection of organellar genomes in genomic sequence data (Wu et al. 2015, Wolf et al. 2015), and differences in coverage depth can help diagnose the genomic location of particular sequences, including intragenomic and horizontal transfers. Sequenced plant genomes often display significant quantities of plastome DNA translocated to the mitochondrion (Rice et al. 2013; Park et al. 2014). The rate of sequence evolution is typically much lower in the plant mitochondrial genome compared to the plastid and nuclear genomes (Wolfe et al. 1987, Palmer and Herbon 1988, Drouin et al. 2008). Hence, in a plant lineage that has been nonphotosynthetic for many millions of years and may have lost its plastid genome, the mitochondrial genome is the most likely place to find persistent, “fossilized” genes or gene fragments transferred from the plastome.

*Hydnora visseri* (Hydnoraceae), the focal holoparasitic plant of this study, represents one of the eleven independent lineages of parasitic plants (Barkman et al. 2007). The small and entirely heterotrophic family consists of only two genera, *Hydnora* and *Prosopanche*. Given a

stem group age of 100 MYA, and a crown group age of 54 MYA (the split between the two genera *Hydnora* and *Prosopanche*), Hydnoraceae are among the oldest parasitic lineages (Naumann et al. 2013). Hydnoraceae are different from other parasitic flowering plants in many ways (Visser and Musselman 1989; Bolin et al. 2011), and have been described as the “strangest plants in the world” (Visser and Musselman 1986). The fleshy trap flower and a massive, horizontally growing underground stem whose haustoria connect to the host (Figure 1), are the only remaining plant organs (Bolin et al. 2011; Wagner et al. 2014). The highly modified flowers of Hydnoraceae have three large, sometimes very brightly-colored tepals that emit volatiles reminiscent of rotting flesh and attract and temporarily imprison huge numbers of carrion beetles for their pollination services (Bolin et al. 2009). While the extraordinary flowers of *H. triceps* are strictly subterranean, flowers of most species break through the surface to reproduce; the emerging flowers grow with so much force that they can crack asphalt or concrete (Maass and Musselman 2001). *Hydnora visseri* grows in desert habitats of Namibia and feeds exclusively on *Euphorbia gregaria* and *E. gummifera* (Bolin et al. 2011), while other members of the genus *Hydnora* feed upon a wider range of host plants in the spurge (Euphorbiaceae), legume (Fabaceae), and torchwood (Burseraceae) families (Musselman and Visser 1989; Bolin et al. 2010; Beentje and Luke 2002). In addition to Fabaceae and Euphorbiaceae, *Prosopanche* has a much wider host range, including Anacardiaceae, Apiaceae, Aquifoliaceae, Asteraceae, Amaranthaceae, Malvaceae, Rhamnaceae, and Solanaceae (Cocucci and Cocucci 1996). Hydnoraceae are placed in the order Piperales (Nickrent et al. 2002; Naumann et al. 2013), with their closest relatives among the first successive branches of living angiosperms, commonly referred to as the “basal angiosperms” (Jansen et al. 2007). Given the age of Hydnoraceae and the highly modified morphology following the ancient loss of photosynthesis, this lineage is a potential candidate, along with *Rafflesia*, to have lost the plastid genome entirely (Nickrent et al. 1997). Here we

a) describe the challenges of identifying and annotating the full plastome from genomic

sequence data of *Hydnora visseri*, b) describe the extreme reduction in both size and gene content that goes far beyond the loss of genes related to photosynthesis, and c) discuss the relevance of the *Hydnora* plastome in the context of extreme genome reduction and sequence divergence.

## Material and Methods

### Plant material, DNA extraction and genome sequencing

*Plant material of Hydnora visseri* and *H. longicollis* was collected on private property (Gondwana Cañon Preserve) (Namibian MET Permit No. 1350/2009). The tissue was snap frozen after collection, shipped and stored at -80 °C. Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen) and used for library preparation from *Hydnora visseri* (insert size of 300 bp) for the Illumina HiSeq 2000 in the laboratory of Stephan C. Schuster (Penn State University). One lane of 100 bp X 100 bp paired-end sequence was obtained, yielding 162,683,243 trimmed reads and comprising over 16 GB total DNA sequence.

### Data processing, genome assembly, and read mapping

The genomic raw data were processed using CLC assembly cell beta (Version 4.0.6 for Linux). This program was also used to remove duplicate reads created during the PCR amplification step of library preparation and to trim adapter sequences and low quality bases (<Q20) from the read data. The genomic reads were *de novo* assembled in CLC, with the scaffolding mode accounting for the pre-computed paired-end insert size, producing 135 Mb of assembled genomic sequence data.

To identify any organellar scaffolds, “gene feature” data extracted from 33 plastid and 14 mitochondrial genomes were aligned with BLASTn (e-value 1e-10) against the *Hydnora* genomic assembly. This search included the closest available plastome to *Hydnora*, that of

*Piper* (Piperaceae; Cai et al. 2006), a nonparasitic relative of Hydnoraceae also from the order Piperales (Naumann et al. 2013). This search returned 78 putative organellar scaffolds that were further assembled in Geneious (Version 7.1.2, Biomatters Limited, <http://www.geneious.com>, Kearse et al. 2012) using the “*De novo* assemble” tool, reducing the number of scaffolds to 58.

Having identified a total of 58 scaffolds with BLAST alignments to organelle genes (plastid or mitochondrial) we next sought to characterize the relative sequence depths (stoichiometries) of each contig with and without detected organelle sequences. The read mapping was performed with CLC Cell (version beta 4.0.6 for Linux) using “ref-assemble”, and read densities were then visualized using R (R 3.2.0 GUI 1.65 Mavericks build (6931)) and the “RColorBrewer” package. Contigs containing positive BLAST hits to mitochondrial or plastid genes are indicated in red and green, respectively (Figure 2).

One plastid scaffold of length 24,268 bp was identified with very high (~1,400X) average read depth. To see if this scaffold connects to any additional sequences in the assembly, it was used as a query in another BLASTn search. A second scaffold of length 1,650 bp was observed at a similar sequence depth (1,389X). A 50 bp overlap allowed the two high depth fragments to be merged, and closed to form a circle with a short inverted repeat. PCR primers were designed to amplify across all four SC to IR junctions and the 50 bp scaffold joins, confirming a circular structure with an inverted repeat. This circular-mapping DNA molecule represents the complete plastid genome of *Hydnora visseri* (GenBank Accession Number KT970098). In contrast to the plastome, most mitochondrial genes were present on scaffolds of much lower (~40X) depth of coverage. However, a few more plastid and mitochondrial gene fragments were identified on scaffolds at around 2X coverage; these are presumably part of the nuclear genome (Figure S1).

## Annotation

Just two genes were identified on the plastome by the initial BLASTn search (*rrn16* and *rrn23*). To further complete the annotation of the plastid genome, DOGMA (<http://dogma.ccbb.utexas.edu/>, Wyman et al. 2004) was used at different stringencies. Settings less stringent than the default settings (50% sequence identity in protein coding genes and 60% in RNA genes) and an e-value of  $1e^{-5}$  identified thirteen additional genes, including the three tRNAs (Table S1).

Furthermore, four additional alignment tools [(1) Geneious tool “Map to Reference” (Version 7.1.2, Biomatters Limited, <http://www.geneious.com>, Kearse et al. 2012) (2) LASTZ “Align Whole Genomes” (Harris et al. 2010) implemented in Geneious, (3) BWA-MEM version 0.7.8 (Li et al. 2009), and (4) GMAP version 2014-02-28 (Thomas et al. 2005)] were applied to all scaffolds identified in the initial BLASTn search. All of these programs were set up to align angiosperm organellar genes (the same that were used as a query in the initial BLASTn search) to the *Hydnora* organellar scaffolds as a reference sequence. All of those approaches returned different results with respect to genes identified, and the results had to be compared carefully and in some cases adjusted manually to obtain the longest alignments with the fewest gaps. A summary of all identified plastid and mitochondrial genes and gene fragments found with each method is provided in Table S1. With respect to the *Hydnora* plastome, four additional genes were identified with this approach (*rps4*, *rps7*, *ycf1*, and *rrn4.5*). Next, we identified all ORFs larger than 100 bp using Geneious (Version 7.1.2, Biomatters Limited, <http://www.geneious.com>, Kearse et al. 2012), and used tBLASTx and PSI-BLAST in NCBI to assign unannotated ORFs, which identified *rps2*, *rps3*, *rps11*, *rps18*, and *ycf2*. Also, unannotated sections of the plastome were used to query the database using BLASTn, but did not recover any new genes.

To verify and complete the annotation of the plastid genome, DOGMA (<http://dogma.ccbb.utexas.edu/>, Wyman et al. 2004) was used at very low stringencies (25% sequence identity in protein coding genes and 30% in RNA genes) and an e-value of  $1e^{-5}$ . These settings identified 23 out of 24 genes (not including *rrn4.5*). Final annotation (gene boundaries) was based on the identified ORFs for all of the protein coding genes. Short exons for *rpl16* and *rps12* were identified manually by aligning the corresponding *Piper* sequence to *Hydnora*. The resulting annotation was submitted to OrganellarGenomeDRAW (<http://ogdraw.mpimp-golm.mpg.de/>, Lohse et al. 2007, 2013).

#### Amplification of gDNA and cDNA

The structure of the plastid genome of *Hydnora visseri* was validated using PCR of gDNA. All genes found on the *Hydnora visseri* plastid genome, as well as the IR boundaries, were amplified and re-sequenced from genomic DNA of *H. visseri* and *H. longicollis* using custom primers designed from the *H. visseri* plastome sequence.

Transcription of 19 plastid genes was confirmed using RT-PCR (not including the three short tRNAs, *rps18*, and *rrn4.5*). Experimental design for RT-PCR confirmation of *rps12* splicing was modeled after Ems *et al.* (1995) using RNA and DNA inputs and multiple experimental controls. All primers used here are listed in Table S2. Total RNA was extracted from *H. visseri* tepal and *H. longicollis* floral bud tissue using a cetyltrimethylammonium bromide (CTAB) RNA isolation protocol (Chang et al. 1993). Total nucleic acids were divided equally for serial DNase I (Qiagen) and RNase A (Qiagen) treatments. RNA digestions were performed in solution with 300  $\mu$ g RNase A at 37 °C for one hour. DNA digestions were performed following Appendix C of the RNeasy Minelute Clean-up Handbook (Qiagen). DNAs and RNAs were then purified using the DNeasy and RNeasy Mini Kit, respectively. Nucleic acid concentrations were estimated using Qubit High Sensitivity DNA and RNA



assays. 1  $\mu$ g RNA from each of the extracted RNA treatments was reverse transcribed using Maxima First Stand Synthesis Kit (Thermo Scientific).

RT-PCR amplifications were performed using DreamTaq (Thermo Scientific) in an Eppendorf Thermocycler using the following parameters: 5 minutes initial melt (95 °C) followed by 35 cycles of 30 seconds melt (95 °C), 30 seconds annealing (50 °C), 30 seconds extension (72 °C), and a final extension of 10 minutes (72 °C). 3 ng of gDNA and cDNA, as estimated by RNA mass added to cDNA synthesis reactions, and added to each reaction mix. PCR products were run on a 2% agarose gel containing 0.5X SyberSafe Dye (Life Technologies) at 125 V for 1 hour. Images were taken on a Molecular Imager Gel Doc XR system (Bio Rad) and Quantity One (Bio Rad) used for estimation of PCR product sizes with respect to the 1 kb Plus ladder (Life Technologies). PCR product for both *Hydnora* species was purified using MinElute PCR Purification Kit (Qiagen): Purified product was sequenced at GeneWiz.

#### Phylogenetic analyses

19 plastid genes derived from the plastid genome were added to the respective angiosperm-wide alignments published by Jansen et al. 2007. Phylogenetic trees for a concatenated alignment of all 20 genes were calculated in RAxML v7.2.6 (Stamatakis 2006) applying the GTR+G model for the rapid Bootstrap (BS) algorithm that is combined with the search for the best scoring ML tree. 1,000 BS replicates were applied for all analyses. Due to the high sequence divergence of the *Hydnora* sequences, a starting tree for the nonparasitic taxa (Jansen et al. 2007) was used. Using the “-t” function allowed to add the *Hydnora* sequences to the existing tree, that is then optimized under ML (Stamatakis 2006). The phylogenetic trees were formatted with TreeGraph2 (Stöver & Müller 2010).

## Test for relaxed selection of plastid genes

To test for relaxed selection of the *Hydnora* plastid genes, different hypotheses were tested for 14 genes and the concatenated dataset using CodeML implemented in PAML (pamlX v1.2, Xu and Yang 2013, Yang 2007). Since the individual plastid genes do not provide enough phylogenetic information to obtain a correct species tree, the input trees for CodeML were calculated in RAxML (Stamatakis 2006) using a starting tree (“-t” function) that comprises the full sampling (including the two *Hydnora* species this time). The basic model was used to calculate the  $d_N/d_S$  ratio of the background, whereas the branch model was used to calculate the  $d_N/d_S$  ratio of the *Hydnora* branches and the background separately. Significance was tested using the difference of likelihood ratios of both models (background vs. branch model) in a simple chi-square test and with one degree of freedom

(<http://www.socscistatistics.com/pvalues/chidistribution.aspx>). For the genes that were tested to be significant for relaxed selection, a second branch model (“selection”), which allows several  $d_N/d_S$  ratios for branches, was used to identify codons that are under positive selection.

## Results

### Plastids of *Hydnora* produce starch granules

In parasitic plants lacking photosynthesis, there are often questions related to plastid function and the state of decay of the plastid genome. Light microscopic (LM) images of tepal and underground stem transverse sections of *Hydnora visseri* and *H. longicollis* stained with iodine-potassium iodide clearly show several starch grains per cell (Figure 3 A and D). Using polarized light, typically a single starch grain per plastid is observed (Figure 3 B and E). As plastids are the exclusive location for building and storing starch (amyloplasts) in a plant cell, this is clear evidence for the presence of plastids in these extreme heterotrophs.

### Size and structure of the *Hydnora visseri* plastome

The 27,233 bp plastid genome of *Hydnora visseri* is only one sixth the size of the plastome of *Piper cenocladum* (160 kb; Cai et al. 2006), a close photosynthetic relative, and nearly half the size of the plastome of *Conopholis americana* (46 kb), a holoparasitic Orobanchaceae with the smallest potentially functional plastome yet known in parasitic plants (Wicke et al. 2013). The circular plastome of *Hydnora visseri* retains the quadripartite structure typical of most characterized plastomes (Wicke et al. 2011; Jansen and Ruhlman 2012), but with much reduced size (Figure 4, Table 1). The large single copy (LSC) region of 22,751 bp and a very short small single copy (SSC) region of 1,550 bp are separated by two short inverted repeats (IRs), each 1,466 bp in length. Structurally, however, the IR-boundaries have shifted drastically in *Hydnora*. The genes *ycf1*, *rps7*, as well as the four rRNAs are located in the IR in *Piper*, but in *Hydnora* they are part of the LSC. The only two genes in the *Hydnora* SSC are *rps2* and *rpl2*, which are found in the LSC in *Piper*. The IR contains only *trnI*-CAU plus parts of *ycf2* and *rpl2*. As expected, read mapping clearly shows twice the sequencing depth in the IR region (Figure 3).

A direct comparison of the nucleotide sequence of *Piper* and *Hydnora* shows very few colinear regions visible in the dotplot relative the background noise (Figure S2, word size 12 and 100 percent identity, implemented in Geneious (Version 7.1.2, Biomatters Limited, <http://www.geneious.com>, Kearse et al. 2012). Only a LASTZ alignment graph shows a few more clear short lines of identity. That the dissimilarity is due to a very high sequence divergence of *Hydnora* plastome sequences is illustrated by a similar dotplot comparison of *Piper* vs. *Arabidopsis* plastomes (Figure S2). At the same stringency (word size 12, percent identity 100), *Piper* and *Arabidopsis* alignments are easily seen, despite *Hydnora* and *Piper* being members of the Piperales and *Arabidopsis* being a distantly related eudicot. The GC content of the *Hydnora* plastome is 23.7%, which is extremely low compared to 38.3% in *Piper* and 33.2% in *Conopholis*, and is consistent with *Hydnora*'s high sequence divergence (Table 1).

To assess any rearrangements in the *Hydnora* plastome, it was compared to *Piper cenocladum* and *Conopholis americana*. Since sequence divergence is very high in *Hydnora* compared to these two plants, colinearity was visualized based on annotation, using Multi-Genome Synteny viewer (<http://cas-bioinfo.cas.unt.edu/mgsv/index.php>, Figure 5). Both the gene order and the gene orientation are nearly identical compared to *Piper* and *Conopholis* (Figure 5). The only exception being the gene block of *ycf1-rrn5-rrn4.5-rrn23-rrn16-rps12-rps7* that is part of the IR in more typical plastomes, but is found in reverse complement orientation in *Hydnora* compared to *Conopholis* (Figure 5). While *Conopholis* has lost one copy of the IR (Wicke et al. 2013, Figure S2), *Hydnora* has retained a very short IR, but this gene block, originally part of the IR, is not in the IR anymore in *Hydnora*. Hence, this looks like an inversion, but retention of this gene from one side of a once-larger IR is more likely to explain this pattern.

Furthermore, the extreme downsizing of the *Hydnora* plastome has led to a high gene density: 85% of the total length (23,159 bp of 27,233 bp) is occupied by genes, leaving only 5,616 bp of intergenic DNA. In *Piper* the ratio of genic:intergenic DNA is 1.6:1, and in *Hydnora* 3.8:1, which indicates that the density of genes is much greater in the *Hydnora* plastome. The intergenic DNA includes a ca. 600 bp highly repetitive region between the *rps12* 3'-end and *rps7* (Figure S2).

The *Hydnora visseri* plastome encodes just 24 genes

The gene content of the 27 kb plastid genome of *Hydnora visseri* has been greatly reduced to just 24 potentially functional genes: 14 ribosomal protein genes (*rps2*, *rps3*, *rps4*, *rps7*, *rps8*, *rps11*, *rps12*, *rps14*, *rps18*, *rps19*, *rpl2*, *rpl14*, *rpl16*, *rpl36*), four rRNAs (*rrn4.5*, *rrn5*, *rrn16*, *rrn23*), three tRNAs (*trnI*<sup>CAU</sup>, *trnE*<sup>UUC</sup>, *trnM*<sup>CAU</sup>), a single biosynthetic protein coding gene (*accD*), and two protein coding genes of unknown function (*ycf1*, *ycf2*) (Figure 4, Table 2, Table S1). The function of *ycf1* is still under debate (de Vries et al. 2015), although recent experimental evidence in model plants suggested a function of *ycf1* in the TOC/TIC machinery (Kikuchi et al. 2013) and it has been proposed to rename *ycf1* as *tic214* (Nakai et al. 2015). While *ycf2* is the largest plastid coding sequence (if it is not lost e.g. *Epipogium* or *Sciaphila*; Schelkunov et al. 2015, Lam et al. 2015), its function has remained unknown for decades. However, recent evidence shows that the protein encoded by *ycf2* might be important for water regulation (Ruiz-Nieto et al. 2015). Due to high sequence divergence, successful annotation of the *Hydnora* plastome required multiple approaches unlike the relatively straightforward annotation of typical chloroplast genomes. An initial BLASTn search provided only two genes (*rrn16* and *rrn23*). Default settings in DOGMA (Wyman et al. 2004, <http://dogma.ccbb.utexas.edu/>) provided only five protein-coding genes (*accD*, *rps12*, *rpl14*, *rpl16*, and *rpl36*) and the three tRNAs (60% identity cutoff for protein coding genes, 80% identity cutoff for tRNAs, and e-value of 1e<sup>-5</sup>). At very low stringency (25%

identity cutoff for protein coding genes, 30% identity cutoff for tRNAs, and e-value cutoff of  $1e^{-5}$ ) nearly all genes are identified (except the short *rrn4.5* gene). All of the protein-coding genes predict open reading frames (ORFs) that are full length or almost full length compared to the *Piper* plastome, and the longest ORF (*ycf2*) is nearly 5,000 bp long.

The 24 plastid genes present in the *Hydnora* plastome are a perfect subset of those found in *Conopholis* (Figure 6). Genes missing from *Hydnora* that are present and potentially functional in the already drastically reduced *Conopholis* plastome are *clpP*, *matK*, *rpl20*, and *rpl33* plus 15 tRNAs (*trnS*<sup>UGA</sup>, *trnS*<sup>GGA</sup>, *trnS*<sup>GCU</sup>, *trnY*<sup>GUA</sup>, *trnL*<sup>UAG</sup>, *trnH*<sup>GUG</sup>, *trnD*<sup>GUC</sup>, *trnP*<sup>UGG</sup>, *trnF*<sup>GAA</sup>, *trnM*<sup>CAU</sup>, *trnN*<sup>GUU</sup>, *trnQ*<sup>UUG</sup>, *trnR*<sup>UCU</sup>, *trnW*<sup>CCA</sup>, *trnG*<sup>UCC</sup>). Comparing the *Hydnora visseri* plastome to the extremely reduced mycoheterotrophs *Epipogium roseum*, *Epipogium aphyllum* (Schelkunov et al. 2015), and *Sciaphila densiflora* (Lam et al. 2015), *Hydnora* has the fewest genes (Figure 6 and S3). The difference in length of those plastomes is mostly due to the retention of *ycf1* and *ycf2*, as well as the retention of one versus two copies of *rrn16* and *rrn23* in *Hydnora*.

The native origin of the *Hydnora* plastome is phylogenetically verified

Phylogenetic evidence was collected for plastid genes of *H. visseri* and *H. longicollis* using a concatenated dataset of 20 genes (not including the three tRNAs and *ycf1*). Because the *Hydnora* sequences of some genes are extremely divergent compared to the sequences from other angiosperms, a starting plastome tree (Jansen et al. (2007) with the addition of unplaced *Hydnora* sequences) was used for all of the calculations. This approach seemed most reasonable because we were only interested in the placement and apparent divergence of the *Hydnora* sequences from those of photosynthetic relatives. In the phylogeny, *Hydnora* is placed sister to *Drimys* (Canellales), in the magnoliids. A high sequence divergence for *Hydnora* is revealed by an extremely long branch in the phylogram (Figure 7).

## Evidence for functionality of the *Hydnora* plastome

Because open reading frames have been retained for each of the protein coding sequences in *H. visseri*, while at the same time being highly divergent compared to plastid genes of related flowering plants, we posit that all the genes found on the plastid genome are potentially functional.

To obtain additional evidence for the potential functionality of the plastid genome, we next amplified and sequenced 16 protein coding genes and three ribosomal RNA genes on the plastid genome from both genomic DNA (gDNA) and cDNA of two *Hydnora* species (*H. visseri* and *H. longicollis*, Figure S2, GenBank accessions KT922054-KT922083) using primers derived from the *H. visseri* plastome (Table S2). The gene sequences were nearly identical between *H. visseri* and *H. longicollis*, resulting in 5,109 base pairs of alignable nonambiguous gene sequence in the two species. *Rpl2* is very likely a pseudogene in *H. longicollis* (Figure S4) and was excluded from the alignment. The remaining 4,647 bp alignment shows 97.2% identity (4,515 bp identical sites). The ratio of nonsynonymous and synonymous sites ( $d_N/d_S$  ratio) between the two species is 0.093 (gene-wise ranging between 0 and 0.5599), indicating that the plastid proteome as a whole has been subject to purifying selection in these two closely related holoparasites (Table S3). The  $d_S$  estimates are extremely high, and likely saturated with substitutions for the individual genes on the long branch leading to the two *Hydnora* species ( $d_S$  between 2 and 28, not including the two very short gene alignments of *rpl36* and *rps2*) and also of the concatenated dataset ( $d_S=3.9$ ); leading to a lower  $d_N/d_S$  ratio compared to the background. The lower  $d_N/d_S$  ratio is likely a result of the saturated synonymous divergence plus continued nonsynonymous divergence and may not indicate an increase in the intensity of purifying selection. We found four codons that are significant for positive selection (*accD*, codon 9, Q to V; *rps12*, codon 56, R to E; *rps12* codon 60, F to P; *rpl2*, codon 5, L to N), indicating that the pattern we see here might in part



be adaptive evolution. In the future, sequences from a less closely related member of Hydnoraceae, such as *Prosopanche*, will provide additional insights into the selective constraints of the Hydnoraceae plastome genes.

Positive amplification from cDNA verified active transcription of each of the 16 protein-coding and 3 ribosomal RNA genes in both *Hydnora* species, including the likely pseudogene of *rpl2* in *H. longicollis* (Figure S5). Genomic sequences and their corresponding cDNA sequences were identical (8,231 bp of corresponding sequence), meaning that there was no evidence of RNA editing in the *Hydnora* coding regions.

*Rps12* and *rpl16* are the only intron-containing genes in the *H. visseri* plastome. In *Piper*, *rps12* has three exons (e.g. Cai et al. 2006), where, generally in angiosperms, the first intron is a trans-spliced group II<sup>b</sup> intron, and the second intron is a cis-spliced group II<sup>a</sup> intron (Kroeger et al. 2009). Relative locations of the exons on the plastome are similar in *Hydnora*. However, comparison of the gDNA and cDNA sequences in *Hydnora* indicates trans-splicing of the first intron, but no splicing of the 230 bp second intron, whose homolog is cis-spliced in *Piper* (Figure S6). This is surprising since the exons potentially encode a full-length *rps12* open reading frame. If it is true that the second and third exons of this gene are not brought together in mature transcripts, the third exon would be out of reading frame due to the length of the intron. Then the *rps12* sequence may be a very recent pseudogene. For *rpl16* it was not possible to obtain splicing evidence since the 5'-exon of this gene is only nine bp long, which was too short for placing a primer. While *rpl2* has an intron in most flowering plant lineages, particularly in the "basal angiosperms", including *Piper* (Cai et al 2006), this intron is absent from *Hydnora*.

Only three predicted tRNA genes were detected in the *Hydnora* plastome. All three can be folded into characteristic stem-loop structures essentially identical to their homologs in *Piper*

and more distantly related angiosperm species (Figure S7). The structures of the three tRNAs on the plastid genome were calculated using tRNA-Scan (<http://lowelab.ucsc.edu/tRNAscan-SE/>) and redrawn in RNAfold (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>) to obtain higher resolution figures. The predicted anticodons (*trnI*<sup>CAU</sup>, *trnE*<sup>UUC</sup>, *trnfM*<sup>CAU</sup>) are unaltered in *Hydnora*. Compared to *Piper*, *Hydnora* shows one compensatory mutation in the stem of *trnI*<sup>CAU</sup>, and three in *trnfM*<sup>CAU</sup>, all of which retain the stem-loop structures. Two of the three identified tRNAs have a single bp indel compared to their homologs in *Piper*, but in both cases, the indel is located in the variable loop and may not affect functionality (Figure S7). *TrnI*<sup>CAU</sup> and *trnfM*<sup>CAU</sup> have the same anticodon, but *trnI*<sup>CAU</sup> has been shown to be post-transcriptionally modified to AUA (Alkatib et al. 2012).

## Discussion

### The minimal plastid genome of *Hydnora visseri*

The plastid genome of *Hydnora visseri*, a plant belonging to one of the most ancient parasitic angiosperm lineages (Naumann et al. 2013), shows extreme reduction in both size and gene content. The retention of only 24 genes encoded in the plastome and the loss of 89 genes compared to the close photosynthetic relative *Piper cenocladum* (Cai et al. 2006), makes *Hydnora* the most minimal plastome sequenced to date, with respect to gene number, yet multiple lines of evidence suggest that it remains functional. The very long branch of *Hydnora* in the phylogram based on 20 plastid genes indicates a very high base substitution rate that is apparently even greater than in the also highly divergent plastomes of *Sciaphila* (Lam et al. 2015) or the Corsiaceae (Mennes et al. 2015). Although extremely divergent, all 17 protein-coding sequences encode potentially full-length open reading frames, the sequences are highly similar and have experienced purifying selection between two related *Hydnora* species, and transcripts are detected for all 19 tested genes. Compensatory mutations help maintain stem-loop structures in conserved tRNA genes. While the retained genes are dramatically divergent, the gene order is remarkably colinear with *Piper*, indicating that deletions have clearly occurred at a much higher rate than inversions in the plastome of *Hydnora* and its ancestors. This strong bias of deletions being much more numerous than inversions or other changes in gene order has also been observed in the highly reduced plastomes of holoparasitic Orobanchaceae (Wolfe et al. 1992; Wicke et al. 2013) and *Cuscuta* (McNeal et al. 2007; Funk et al. 2007).

Over the past decade, the number of sequenced plastomes of parasitic and mycoheterotrophic plants has increased significantly. Plastomes representing various evolutionary stages leading to and following complete heterotrophy show that similar patterns of gene loss and size

reduction have occurred in both parasitic plants and mycoheterotrophs (Wolfe et al. 1992; McNeal et al. 2007; Funke et al. 2007; Wickett et al. 2008; Delannoy et al. 2011; Logacheva et al. 2011; Barrett and Davis 2012; Wicke et al. 2013; Li et al. 2013; Uribe-Convers et al. 2014; Logacheva et al. 2014; Barrett et al. 2014; Schelkunov et al. 2015, Lam et al. 2015). Independent evidence from multiple taxonomic lineages suggests that the evolution of plastid decay seems to follow a general pattern (Barrett et al. 2012 and 2014; Wicke et al. 2013) associated with the reduction and eventual loss of photosynthetic constraints occurring in both groups with an increase of heterotrophic dependence (Lemaire et al. 2011; Barrett et al. 2014). The high degree of plastome reduction found in the ancient holoparasite *Hydnora* fits this pattern to an extreme, since the total coding capacity of *Hydnora* is the smallest yet observed in a potentially functional plastome.

The typical quadripartite structure of plastid genomes (a large inverted repeat separating two single copy regions) is conserved in most seed plants (Palmer, 1985). Exceptions have been reported in a few plant lineages, including Geraniaceae (Guisinger et al. 2010a), Poaceae (Guisinger et al. 2010b), *Vaccinium* (Fajardo et al. 2013), *Arbutus unedo* (Martinez-Alberola et al. 2013), Fabaceae (Cai et al. 2008), Pinaceae and cupressophytes (Wu et al. 2011). However, nonphotosynthetic plants, which represent a small fraction of angiosperm species, possess remarkably varied plastomes, with structures including large IRs and only one single copy region (*Epipogium aphyllum*; Schelkunov et al. 2015), or a very small IR (*Epipogium roseum*; Schelkunov et al. 2015), to the complete loss of one IR copy (*Conopholis americana*; Wicke et al. 2013). In the *Hydnora visseri* plastid genome, all three plastome regions are retained, but each has been drastically reduced in size. The extreme contraction in size of the IR of *Hydnora* (to ca. 1.5 kb compared to 27 kb in *Piper*) has led to relocation of the genes that are located in the IR in Piperales (and possibly also in the immediate ancestors of *Hydnora*) to mainly the LSC. The gene order has remained mostly unaltered. The retention of

the IR in *Hydnora*, although small, supports the hypothesis that the IR might be important for stabilizing and retaining the plastome over tens of millions of years (Palmer and Thompson 1982; Perry and Wolfe 2002; Maréchal and Brisson 2010).

Entire classes of genes that are commonly pseudogenized or lost during or soon after the transition to the heterotrophic lifestyle are entirely missing from the plastome of *Hydnora*: NADH dehydrogenase (*ndh* genes), ATP synthase (*atp* genes), RNA polymerase (*rpo* genes), photosystem (*psa* and *psb* genes), and cytochrome related genes (*pet* genes). In *Hydnora*, the plastid genome reduction has gone far beyond that seen in assembled plastomes of most other heterotrophic plants. Only ribosomal proteins, ribosomal RNAs, some housekeeping genes, and three tRNAs genes are retained in the *Hydnora* plastome. The four ribosomal RNAs retained in *Hydnora* have also been found in all other plastomes and each is expected to be functionally essential (Wicke et al. 2013, Barrett et al. 2014, Schelkunov et al. 2015, Lam et al. 2015).

The plastomes of photosynthetic plants typically contain 21 ribosomal protein-coding genes, and at most, a handful are missing from nonphotosynthetic plants (Wicke et al. 2013; Barrett et al. 2014). The retention of 14 ribosomal protein genes in the *Hydnora* plastome is similar to the very reduced plastomes of other parasitic or mycotrophic plants where 15 (*Sciaphila*), 16 (*Conopholis*, *Epipogium*) and 19 (*Epifagus*) are retained. The ribosomal protein genes from *Hydnora* are a perfect subset of those from *Conopholis*, where *rpl20* and *rpl33* are retained in addition to those retained in *Hydnora*. To date, *rpl20* has previously been identified as retained in all functional plastomes, and *rpl33* is only reported missing from the plastomes of the mycoheterotrophic orchids (Dellanoy et al. 2011; Schelkunov et al. 2015, Lam et al. 2015). Unless functional ribosomes can be assembled from a slightly smaller number of ribosomal proteins, the absence of seven ribosomal protein genes from the *Hydnora* plastome suggests that these proteins may be imported into the *Hydnora* plastid for ribosome assembly.

This would imply either that these genes have been functionally transferred to another genomic compartment, or that the missing components have been replaced by proteins normally functioning in the mitochondrial or nuclear ribosomes.

The retention of only three of 30 plastid-encoded tRNAs (*trnI*<sup>CAU</sup>, *trnE*<sup>UUC</sup>, *trnfM*<sup>CAU</sup>) is many fewer than what is expected for a minimal functional plastome (Lohan and Wolfe 1998) and is the smallest set of plastid tRNAs that has ever been observed. In comparison, six are retained in the extremely reduced plastomes of *Epipogium aphyllum* and *Sciaphila densiflora* (Schelkunov et al. 2015, Lam et al. 2015) and 14 in *Conopholis* (Wicke et al. 2013). It has been discussed previously, and demonstrated with computer simulations, that some tRNAs could escape deletion by chance because of their small size and only moderate sequence divergence from an autotrophic ancestor (Lohan and Wolfe 1998). Their characteristic cloverleaf structure of tRNAs is highly conserved and is sensitive to mutations, especially in the stem regions. This is possibly the case in Orobanchaceae, as it is a rather young parasitic plant family (20 MY old, including numerous photosynthetic members; Naumann et al. 2013). Hydnoraceae, however, is an ancient parasitic family, with at least 54 MY to over 90 MY of evolution as a holoparasite (Naumann et al. 2013); retention of a plastome by chance becomes more and more unlikely over time, especially considering the extreme downsizing and condensation of the plastome that *Hydnora* has experienced. Additionally, plastomes sequenced to date retain the same set of three tRNAs found in *Hydnora* (*trnI*<sup>CAU</sup>, *trnE*<sup>UUC</sup>, and *trnfM*<sup>CAU</sup>), suggesting an essential function for all three tRNAs. As proposed by Howe and Smith (1991), *trnE* has a dual function in plastid biology (tetrapyrrole biosynthesis and protein biosynthesis), and this could be the reason why it cannot be replaced by its cytosolic counterpart making it an essential plastid-encoded gene. Isoleucine and Methionine, both encoded by two tRNAs (*trnI*<sup>CAU</sup> and *trnI*<sup>GAU</sup>, as well as *trnfM*<sup>CAU</sup> and *trnM*<sup>CAU</sup>), seem to be

essential for any plastome. It has been shown that for each of these two amino acids, at least one tRNA has to be retained (Alkatib et al. 2012).

As many as four plastid protein coding genes were proposed to be essential for a minimal plastome (*ycf1*, *ycf2*, *accD*, *clpP*) (based on *Epifagus virginiana*, Wolfe et al. 1992). Three of these (*ycf1*, *ycf2*, and *accD*) are retained in *Hydnora* and in most other plant plastomes (Figure S4) though one or more of these genes has been lost on occasion from nonphotosynthetic or even photosynthetic plastomes (Wicke et al. 2011; Barrett et al. 2014; Straub et al. 2011a). The caseinolytic protease encoded by *clpP*, which is part of the stromal proteolytic machinery (Adam and Clarke 2002), has been lost from *Hydnora*. *ClpP* is retained even in the most reduced plastomes of nonphotosynthetic plants sequenced to date (Delannoy et al. 2011, Wicke et al. 2013, Schelkunov et al. 2015, Lam et al. 2015). In photosynthetic plants, *clpP* has not been found in *Scaevola* and *Passiflora* (Jansen et al. 2007); it is a pseudogene in *Asclepias* (Straub et al. 2011a), *Monsonia* and *Geranium* (Geraniaceae, Guisinger et al. 2011), *Trachelium* (Haberle et al. 2008), *Arbutus* (Martínez-Alberola et al. 2013), and may encode a nonfunctional protein in *Acacia* (Williams et al. 2015). In those cases, it is possible that a nuclear-encoded homolog is transported into the plastid and functionally compensates for the missing or nonfunctional protease (Williams et al. 2015). Making imported proteins functional seems essential for any plastid, especially in holoparasitic plants where gene products might be retrieved from the host and act as substitutes for plastid-encoded proteins. A functional transfer of *clpP* to the nucleus in *Hydnora* is unlikely, since both the genome (albeit only 2X coverage) and a thorough transcriptome sequence (J. Naumann, unpublished) have been screened for plastid genes at different stringencies. Since there is no evidence for *clpP* in *Hydnora* at all, some other protein may have adapted to serve the essential functions of *clpP* in the plastid.



The beta-carboxyl transferase subunit of acetyl-CoA carboxylase (*accD*) as well as *ycf1* and *ycf2* are present and potentially functional in *Hydnora*. *Ycf2*, in particular, is the longest plastid gene in most plant plastomes (6,945 bp in *Piper*). Although it has an extremely high sequence divergence in *Hydnora*, it encodes a long ORF of 4,920 bp, which would be virtually impossible to be retained by chance during tens of millions of years of heterotrophic evolution and considerable sequence divergence (Leebens-Mack and dePamphilis 2002). The shorter total plastome sequence of *Epipogium roseum* and *Sciaphila densiflora* (Schelkunov et al. 2015, Lam et al. 2015) as compared to *Hydnora* is mostly due to the loss of *ycf1*, and *ycf2*. The absence of these two genes appears to be a common pattern in the extremely reduced mycoheterotrophs (Schelkunov et al. 2015, Lam et al. 2015), but not in the sequenced parasitic plants (Wicke et al. 2013), including *Hydnora*.

A comparison of ten plastid genomes from the Orobanchaceae additionally suggests an essential function of *matK* in that parasite lineage, because genes containing group II<sup>A</sup> introns are retained in the plastomes of that family of parasites (Wicke et al. 2013). On the other hand, *matK* has been lost in *Cuscuta obtusifolia*, *Cuscuta campestris*, and *Cuscuta obtusifolia* (Funk et al. 2007, McNeal et al. 2007, Braukmann et al. 2013) following the progressive and eventually complete loss of all group II<sup>A</sup> introns from *Cuscuta* (McNeal et al. 2007). Parallel loss of *matK* and group II<sup>A</sup> intron-containing genes has occurred in *Rhizanthella gardneri* (Delannoy et al. 2011). Maturase K (MATK) is an enzyme that is required for group II<sup>A</sup> intron splicing of several plastid genes. It is retained in most reduced plastomes, although especially in the genus *Cuscuta* many cases of a pseudogenized *matK* have been reported (Braukmann et al. 2013). If all seven plastid genes containing group II<sup>A</sup> introns (*trnV*<sup>UAC</sup>, *trnI*<sup>GAU</sup>, *trnA*<sup>UGC</sup>, *trnK*<sup>UUU</sup>, *rpl2*, *rps12*, *atpF*, Zoschke et al. 2010) or the introns themselves were lost, *matK* would no longer be required (McNeal et al. 2007). In *rps12*, the first intron is a group II<sup>B</sup>-intron, and the second is a group II<sup>A</sup>-intron, being dependent on MATK for splicing.

Amplification evidence of *rps12* shows that the group II<sup>A</sup>-intron is not being spliced out from *Hydnora* transcripts, which is in accordance with the absence of *matK* from the *Hydnora* plastome. This implies that this gene is being mis-spliced and is potentially non-functional (i.e. pseudogene), or it is spliced by another enzyme that is not plastid encoded. While all genes on the plastome of the white albobriars barley mutants are transcribed, their translation is deficient due to lack of chloroplast ribosomes. It has been shown that the lack of MATK results in an immature mRNA of *rps12*, where the group II<sup>A</sup> intron is not being spliced out (Hübschmann et al. 1996). In a green plant, where many genes require MATK to produce mature mRNAs, the loss of this protein would likely be lethal. In *Hydnora*, the second *rps12* intron is the only MATK dependent intron left, and thus proper splicing is disrupted in only one gene as opposed to seven. A similar pattern is found in plastomes of both *Epipogium* species (Lam et al. 2015). Both plastomes have lost *matK*, but have retained group II<sup>A</sup> introns: the *rpl2* intron (both species), and the second intron of *clpP* (*Epipogium roseum*) are still retained. Loss of *matK* prior to the loss of one or more genes with intron sequences that depend on splicing by MATK, is an alternative scenario from that proposed by McNeal et al. (2009) to explain the eventual loss of both *matK* and group II<sup>A</sup> intron containing genes. Whether *matK* is lost prior to the final group II<sup>A</sup> intron loss (as suggested in *Hydnora*) or lost simultaneously to or after the loss of all group II<sup>A</sup> introns (McNeal et al. 2009) could depend on the particular lineage in question, but the ultimate outcome – loss of both introns and maturase – would be identical.

*Rpl2* is another gene that contains a group II<sup>A</sup> intron in most angiosperms, but one that has also lost its intron several times in different angiosperm lineages independently (Downie et al. 1991). While *rpl2* seems functional in *H. visseri*, it appears to be a pseudogene in *H. longicollis* because of several indels throughout the gene that lead to frameshifts and numerous inferred stop codons. Both *rps12* (both species) and *rpl2* (only *H. longicollis*) may

be in early stages of pseudogenization. They are transcribed in both *H. visseri* and *H. longicollis*, but it is unlikely that they could be translated into functional proteins. In some Orobanchaceae, *rbcL* is in early stages of pseudogenization (specifically in *Hyobanche*). The *rbcL* pseudogene was shown to be transcribed, but active RuBisCo enzyme was detected in some tissues and was hypothesized to be parasitized host enzyme (Randle and Wolfe 2005).

When the *Epifagus* plastome was first mapped, sequenced, and discussed (dePamphilis and Palmer 1990; Wolfe et al. 1992), it was hypothesized that a minimal plastome would require the functional retention of at least one gene required for a retained plastid-specific process, plus any nonexpendable machinery for its expression (Wolfe et al. 1992). This hypothesis has persisted through many sequenced plastomes of nonphotosynthetic plants and still holds in light of the *Hydnora* plastome. In addition to the dual function of *trnE* (Howe and Smith 1991), at least some ribosomal protein coding genes and plastid ribosomal RNAs may be retained because they cannot be transferred to the nucleus due to interference with their cytosolic equivalents (Howe and Smith 1991; Barbrook 2006). A third hypothesis to explain retention of some plastid genes (and therefore the plastid genome) is that plastid encoding is required for correct regulation of plastid gene expression based on redox balance (Allen et al. 2003). As opposed to earlier stages in the evolution towards holoparasitism, other than retention of rDNAs and some ribosomal protein and tRNA genes in all of the reduced plastomes, there seems to be no universal pattern to the loss or retention of the very last few genes such as *accD*, *clpP*, *ycf1*, and *ycf2*. In different plant lineages, losses of one or more of these “potentially essential” genes have been reported repeatedly (reviewed in Wicke et al. 2011) and may depend upon whether nuclear or mitochondrial homologs can substitute for the loss of function of plastid copies in specific lineages of plants.

## The possible loss of plastid genomes re-visited

The holoparasitic *Rafflesia lagascae* is a recently reported case of a potentially lost plastid genome in a flowering plant (Molina et al. 2014). This remarkable claim reopens the debate as to whether or not a plastid genome could be lost in plants (dePamphilis and Palmer 1990, Wolfe et al. 1992, Nickrent et al. 1997, Race 1999, Bungard et al. 2004, Barbrook et al. 2006, Krause 2008, Wicke et al. 2011, Janouskovec et al. 2015). Whenever the question of the loss or retention of a plastid genome is raised in a specific plant, first and foremost, the presence or absence of any particular type of plastid should be investigated. In *Hydnora visseri* we find amyloplasts, plastids that are specialized for starch synthesis and storage. The storage of large amounts of starch might be important for *Hydnora* for floral thermogenesis (Seymour et al 2009). An additional possibility is that the starch might help *Hydnora* outlast recurrent periods of extreme drought in the desert habitat where it lives, because when the stored starch is degraded into monomers, water can be derived by oxidation of glucose in a subsequent reaction (Spoehr 1919).

The unicellular algae *Polytomella* appear to have also retained amyloplasts, but strong evidence was presented that the plastome has been lost from *Polytomella* (Smith and Lee 2014). In *Rafflesia*, the situation is less clear. While plastid-like structures have been observed, they are not reported to contain starch (Molina et al. 2014). On a cellular level, whether or not the plastid-like structures in *Rafflesia* are derived plastids could be further explored with fluorescence labeling of known mitochondrial and nuclear-encoded plastid markers or fluorescence staining of the organellar membranes. The endophytic life style of *Rafflesia* could possibly reduce the spectrum of required plastid types, as well as enable the drastic evolutionary step of a complete plastome loss. If it is true that *Rafflesia* has lost its plastid genome, but retained its plastids, it has apparently retained function(s) other than starch storage.

However, it remains arguable whether or not *Rafflesia* has lost its plastid genome. If *Rafflesia*'s gene sequences were highly divergent from available plastid genome sequences, as we have shown to be the case for *Hydnora*, there is a chance it could have escaped detection by all of the approaches used in Molina et al. (2014) (mapping scaffolds to a photosynthetic reference, BLASTn to plastid genomes, and Hidden Markov Models of plastid gene alignments). Due to the reduced size and gene content, as well as the high sequence divergence and compositional bias of coding genes, finding plastid sequences in the *Hydnora visseri* assembly was extremely challenging. Basic similarity-based approaches did not serve to identify plastid genes as they would for the vast majority of plants, where the plastid genome is very straightforward to extract from genomic sequence assemblies (Straub et al. 2011b). The identification and annotation of the *Hydnora* plastid genome was a long process that required multiple approaches, where especially the relative read depths for the three genomic compartments were found to be valuable evidence for identifying organellar genomes.

Even in low coverage genomic data (Straub et al. 2011a, Wolf et al. 2015), the plastid and the mitochondrial genomes are expected to be captured at distinct stoichiometries in the sample (Bock 2007, Straub et al. 2011b; Wolf et al. 2015). Usually, there are tens to hundreds of mitochondrial genomes per cell, but thousands of plastomes, which will typically show a higher stoichiometry for a functional plastome (Straub et al. 2011b). Nonphotosynthetic or senescent tissues with reduced photosynthetic activity, however, can show a decreased plastome copy number (Fulgosi et al. 2012) and thus, show a reduced read depth of the plastome in the genomic sample (Bowman & Simon 2013). If the plastome cannot be identified by its stoichiometry in the genomic assembly, the mitochondrial genome is a potentially important place to carefully look for old plastid gene “fossils”. As gene transfer from the plastid to the mitochondrion is commonly discovered in flowering plants, and plant

mitochondrial genes generally show a very low substitution rate, the origin of integrated genes, and sometimes even ancient transfers, can be tracked back to their plastid origins (Wolfe et al. 1987, Mower et al. 2007, Rice et al. 2014).

## Conclusion

The plastid genome of *Hydnora visseri* shows a unique combination of features: an extreme downsizing and gene reduction, especially of the tRNAs, and an extreme sequence divergence and base compositional bias, while the retained genes show multiple indications of probable functionality. This suggests the following evolutionary scenario for the plastid genome in nonphotosynthetic plants. First, in the “degradation stage I”, non-essential and photosynthesis related genes are pseudogenized successively, followed by complete loss of those genes. The order of gene loss follows a recurring pattern in the different lineages of hemiparasitic and nonphotosynthetic plants, particularly observable in the Orobanchaceae and the Orchidaceae, where plastomes with various degrees of reduction have been examined (e.g. Barrett et al. 2012, Wicke et al. 2013, Barrett et al. 2014). Second, in the “stationary stage” only genes required for nonphotosynthetic functions are retained, the rate of gene loss is much slower, and pseudogenes are expected to be rarely produced. At this stage, further gene loss is likely to be dependent upon the ability of imported or substitute proteins to serve any continuing required function in the nonphotosynthetic plastid. Alternatively, successful functional transfers of genes into the nucleus or mitochondrion, with a transit peptide to direct the protein back into the plastid, could allow additional genes to be lost from the plastome. Such events of functional gene transfer in green plant lineages are rare (Baldauf and Palmer 1990, Martin et al. 1998). Thus, this stage can potentially last much longer than the degradation stages, although the duration of the stationary stage may be lineage specific and depend on many factors. The retained plastid genes, however, continue to evolve, sometimes with a relatively high rate of net mutation after repair, hence they show a high level of sequence



divergence after tens of million of years. If the last essential plastid gene is lost or functionally replaced with a nuclear or mitochondrial copy, the plastome would become unnecessary; this would mark the third, or “degradation stage II”. If this occurs, then any remaining genes that have been maintained only to facilitate the expression of an essential gene will be pseudogenized and eventually lost; this would make a remnant plastome in this stage even harder to detect. Finally, in the “absent” stage, the plastome is completely lost, except for small fragments that may reside in other parts of the genome, and those genes functionally transferred to another genomic location whose gene products still function in the plastid. In the case of Rafflesiaceae, they either have an extremely reduced, even more divergent plastome than *Hydnora* (late stage two or stage three), or this lineage has entirely lost its plastome (stage four). The retained short and low coverage plastid fragments of *Rafflesia* and *Polytomella* (Molina et al. 2014, Smith and Lee 2014, respectively) remain to be characterized in more detail. The minimal, but functional *Hydnora* plastome, being well into stage two, helps us understand how diminished a plastome can be while still retaining functionality.

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## Tables

Table 1. Comparison of the plastid genomes of <i>Hydnora visseri</i> and <i>Piper cenocladum</i>		
	<i>Piper</i>	<i>Hydnora</i>
Size (bp)	160,624	27,233
Genic (bp)	100,645	21,617
Intergenic (bp)	59,979	5,616
Percentage genic	62.66%	79.38%
Percentage intergenic	37.34%	20.62%
LSC length (bp)	87,668	24,114
SSC length (bp)	18,878	1,550
IR length (bp)	27,039	1,466
Number of genes (unique genes)	130 (113)	25 (24)
Number of genes duplicated in IR	17	1
Number of genes with introns	18	3
GC content	38.3%	23.4%

Table 2. Summary of plastid genes present and absent in *Hydnora*.

Present on <i>Hydnora</i> plastid genome		Deleted from <i>Hydnora</i> plastid genome			
Ribosomal RNA genes					
<i>rrn16</i>	<i>rrn4.5</i>				
<i>rrn23</i>	<i>rrn5</i>				
Transfer RNA genes					
<i>trnE</i> -UUC	<i>trnA</i> -UGC	<i>trnK</i> -UUU	<i>trnQ</i> -UUG	<i>trnT</i> -GGU	
<i>trnfM</i> -CAU	<i>trnC</i> -GCA	<i>trnL</i> -CAA	<i>trnR</i> -ACG	<i>trnT</i> -UGU	
<i>trnI</i> -CAU	<i>trnD</i> -GUC	<i>trnL</i> -UAA	<i>trnR</i> -UCU	<i>trnV</i> -GAC	
	<i>trnF</i> -GAA	<i>trnL</i> -UAG	<i>trnS</i> -GCU	<i>trnV</i> -UAC	
	<i>trnG</i> -GCC	<i>trnM</i> -CAU	<i>trnS</i> -GGA	<i>trnW</i> -CCA	
	<i>trnH</i> -GUG	<i>trnN</i> -GUU	<i>trnS</i> -UGA	<i>trnY</i> -GUA	
	<i>trnI</i> -GAU	<i>trnP</i> -UGG			
Ribosomal protein genes					
<i>rpl2</i>	<i>rps7</i>		<i>rpl20</i>		
<i>rpl14</i>	<i>rps8</i>		<i>rpl22</i>		
<i>rpl16</i>	<i>rps11</i>		<i>rpl23</i>		
<i>rpl36</i>	<i>rps12</i>		<i>rpl32</i>		
<i>rps2</i>	<i>rps14</i>		<i>rpl33</i>		
<i>rps3</i>	<i>rps18</i>		<i>rps15</i>		
<i>rps4</i>	<i>rps19</i>		<i>rps16</i>		
Other genes					
<i>accD</i>		<i>clpP</i>	<i>ycf15</i>		
<i>ycf1</i>		<i>matK</i>	<i>ycf3</i>		
<i>ycf2</i>			<i>ycf4</i>		
RNA polymerase					
		<i>rpoA</i>	<i>rpoC1</i>		
		<i>rpoB</i>	<i>rpoC2</i>		
Photosynthetic and chlororespiratory genes					
	<i>atpB</i>	<i>ndhA</i>	<i>infA</i>	<i>psbD</i>	
	<i>atpE</i>	<i>ndhB</i>	<i>ccsA</i>	<i>psbE</i>	
	<i>atpF</i>	<i>ndhC</i>	<i>cemA</i>	<i>psbF</i>	
	<i>atpH</i>	<i>ndhD</i>	<i>rbcL</i>	<i>psbH</i>	
	<i>atpI</i>	<i>ndhE</i>	<i>psaA</i>	<i>psbI</i>	
	<i>petA</i>	<i>ndhF</i>	<i>psaB</i>	<i>psbJ</i>	
	<i>petB</i>	<i>ndhG</i>	<i>psaC</i>	<i>psbK</i>	
	<i>petD</i>	<i>ndhH</i>	<i>psaI</i>	<i>psbL</i>	
	<i>petG</i>	<i>ndhI</i>	<i>psaJ</i>	<i>psbM</i>	
	<i>petL</i>	<i>ndhJ</i>	<i>psbA</i>	<i>psbN</i>	
	<i>petN</i>	<i>ndhK</i>	<i>psbB</i>	<i>psbT</i>	
			<i>psbC</i>	<i>psbZ</i>	

Plastid genes residing on the mitochondrial genome as pseudogenes are indicated in bold.

Fig. 1. *Hydnora visseri* A. Flower B. Underground stem (dark) connected to host plant *Euphorbia gregaria* (light) and a close-up. C. Flower bud of *H. visseri* in foreground next to shovel and *Euphorbia gregaria* stems in background D. Cross section of *H. visseri* underground stem.

Fig. 2. Plot of read depths relative to scaffold length. Cyan circles are all scaffolds from the genomic assembly creating darker spots at greater read depths. Scaffolds containing BLAST hits to plastid genes are overplotted by green filled circles (top). Scaffolds containing BLAST hits to mitochondrial genes are overplotted by red filled circles (bottom). All scaffolds at around 40X coverage that contain plastid gene fragments also contain mitochondrial genes. This indicates plastid sequences that have migrated into the mitochondrial genome. The two green filled circles at around 1,400X coverage (filled black arrows) contain only plastid genes and comprise the *Hydnora* plastid genome. The remaining cyan circles correspond to the nuclear genome. The remaining red and green filled circles are presumably mitochondrial and plastid genes that are located in the nucleus.

Fig. 3. Light microscopy of two different starch-containing tissues of *Hydnora visseri*. A. Section of tepal, starch grains in cells stained with iodine-potassium iodide. B. Section of tepal, showing starch grains under polarized light; inset: enlarged starch grains. C. Transverse section of underground organ; stained with Astrablue-safranin; 5-merous organization of vascular system is visible. D. Starch grains in the underground organ; stained with iodine-potassium iodide. E. Starch grains and vascular bundle in underground organ under polarized light.

Fig. 4. Map of the plastid genome of *Hydnora visseri*. Genes are color coded according to the legend, which indicates functional groups. The inner ring illustrates the boundaries of the large single copy (LSC) and small single copy (SSC) regions, separated by the two copies of

the inverted repeat (IRA, IRB). The innermost ring shows the GC content across the plastid genome.

Fig. 5. Maps of plastid genomes showing colinearity. The two circles show annotation based syntenic regions between *Hydnora* and *Piper* and respectively *Hydnora* and *Conopholis* (generated in mGSV, <http://cas-bioinfo.cas.unt.edu/mgsv/>). At the bottom there is a comparison between all three species. Gene order is co-linear with the exception of an inversion of a large gene block between *Hydnora* and *Conopholis* that originally stems from opposite IRs.

Fig. 6. Gene content of highly reduced plastid genomes of seven nonphotosynthetic plants. The gene set shown here excludes gene categories that are missing in most of these extremely reduced plastomes. Black boxes indicate a present gene, white boxes indicate absent gene, and gray boxes indicate a pseudogene. A more comprehensive version of this figure including the full plastome gene set and including the available complete plastomes of hemiparasitic or nonphotosynthetic plants, as well as close photosynthetic relatives, is shown in Figure S4.

Fig. 7. Maximum likelihood phylogenetic trees of the *Hydnora visseri* plastid genes. A phylogenetic tree has been estimated for a concatenated dataset of 20 plastid genes, based on alignments published by Jansen et al. 2007. The tree was estimated with RAxML (Stamatakis 2006), applying the rapid bootstrapping algorithm (1000 bootstrap replicates). The topology of Jansen et al. 2007 was used as a starting tree (-t function in RAxML). The cladogram (left) verifies a magnoliid origin of the *Hydnora* plastid genes. Bootstrap values are plotted above nodes. The scale of the phylogram is in substitutions per site. The phylogram (right) shows a much higher number of substitutions for the *Hydnora* sequences compared to the other taxa.

Fig. S1. Histogram coverage. The histogram of the *Hydnora visseri* genomic assembly shows a coverage estimate of a 2X genomic read depth. The two graphs differ in the scale of the x-axis.

Fig. S2. Dot matrices of plastid genomes. On the top are self-dot plots, not true to scale, of the *Hydnora*, *Conopholis* and *Piper* plastid genomes (from left to right) with a window size of 12, and absolute identity. Inverted lines indicate inverted repeat copies. The IR in *Hydnora* is much smaller than the *Piper* IR. *Conopholis* has lost the second copy of the IR completely. At the bottom there is the *Hydnora* plastid genome plotted against *Piper* with the same window size (12) and scale. Lines indicate regions with high identity. Below, there are LASTZ alignment graphs of the *Piper* plastid genome against *Hydnora* and *Arabidopsis*, also showing sequence similarity, but reduced noise makes syntenic regions more visible.

Fig. S3. Gene content of plastid genomes of parasitic plants, mycoheterotrophs, and close photosynthetic relatives. Black boxes indicate genes that are present, white boxes indicate genes that are absent, and gray boxes indicate pseudogenes. The annotation is mostly based on the GenBank entries of the respective species. The annotation of both *Orobanche* species is based on Cusimano and Wicke, in press).

Fig. S4. Alignment of *rpl2* in *Hydnora visseri* and *H. longicollis*. The sequence of *H. visseri* is full length, while the sequence from *H. longicollis* is obtained from cDNA. The nucleotide sequence shows four indels leading to frameshifts visible in the corresponding translation below.

Fig. S5. Amplification results of *Hydnora visseri* and *Hydnora longicollis* from cDNA and gDNA. IR boundaries were verified using gDNA from both species.

Fig. S6. Validation of introns present in *rps12*. This figure details the experimental gDNA and cDNA PCR reactions and corresponding controls used in the verification of *rps12* in the two *Hydnora* species, as well as the expected and observed product sizes. The amplification spanning exons 1 and 2 shows cDNA product of a size corresponding to the spliced transcript size with no apparent amplification of gDNA due to the 13 kb distance separating the two exons in the plastome (Figure S5A). gDNA amplification of exon 1 through intron 2 also resulted in a lack of amplification, but did result in cDNA amplification, with the resulting product size indicating an absence of splicing between exons 2 and 3. This evidence was further supported by the equivalent sizes of the gDNA and cDNA products spanning exons 2 and 3. The absence of amplification in the control reactions (with the exception of lane 7 in both species indicating the presence of gDNA in the extracted total RNA and lane 6 in *H. longicollis* indicating an incomplete DNase I digestion) support the conclusion of RNA origin of the amplified transcript as indicated by the presence of product in lane 4 and the absence of product in lane 8.

An alternative gene model in *Hydnora* is illustrated in Figure S5B. In this model the trans-splicing of intron 1 is successfully performed while the cis-splicing of intron 2 has failed. This conclusion is evidenced by cDNA bands of approximately the same size as predicted in the exon1-exon2 spanning amplification, presence of a product in the exon1-intron2 amplification coupled with the absence of a genomic product, and gDNA and cDNA products of equivalent sizes in the exon2-exon3 spanning amplification. This experiment predicts that both *H. visseri* and *H. longicollis* have retained the capacity to trans-splice while losing the capacity for cis-splicing, and further predicts the reduced size of intron 2 in *H. longicollis*. Sequencing of these products have confirmed these predictions with both species showing clear evidence of intron 2 retention, and with *H. longicollis* displaying an intron 2 of 177 bp as compared to 230 bp for *H. visseri*.

Fig. S7. tRNA structures. Comparisons to four other photosynthetic plants show conserved anticodon sequences (in bold) and compensatory mutations (indicated with a red frame) in *Hydnora* that retain conserved stem loop structures, and only minor differences that very likely do not affect the functionality of the tRNAs (insertion or deletion indicated with red filled triangle).



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Figure 1



Figure 2

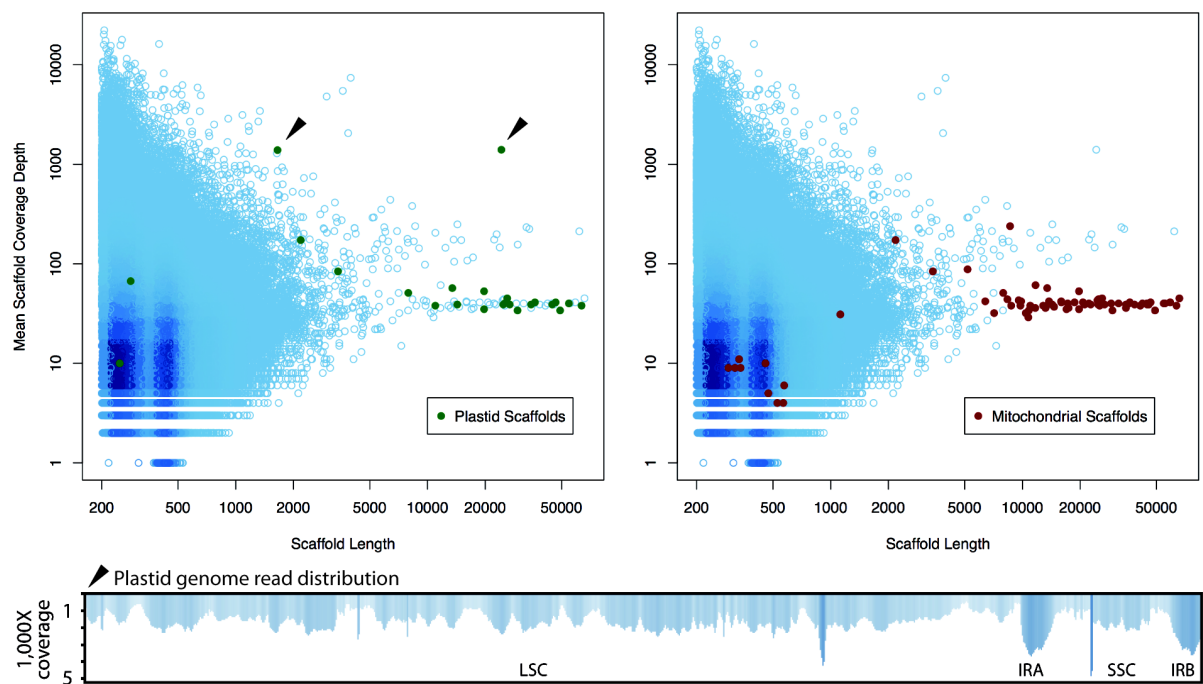


Figure 3

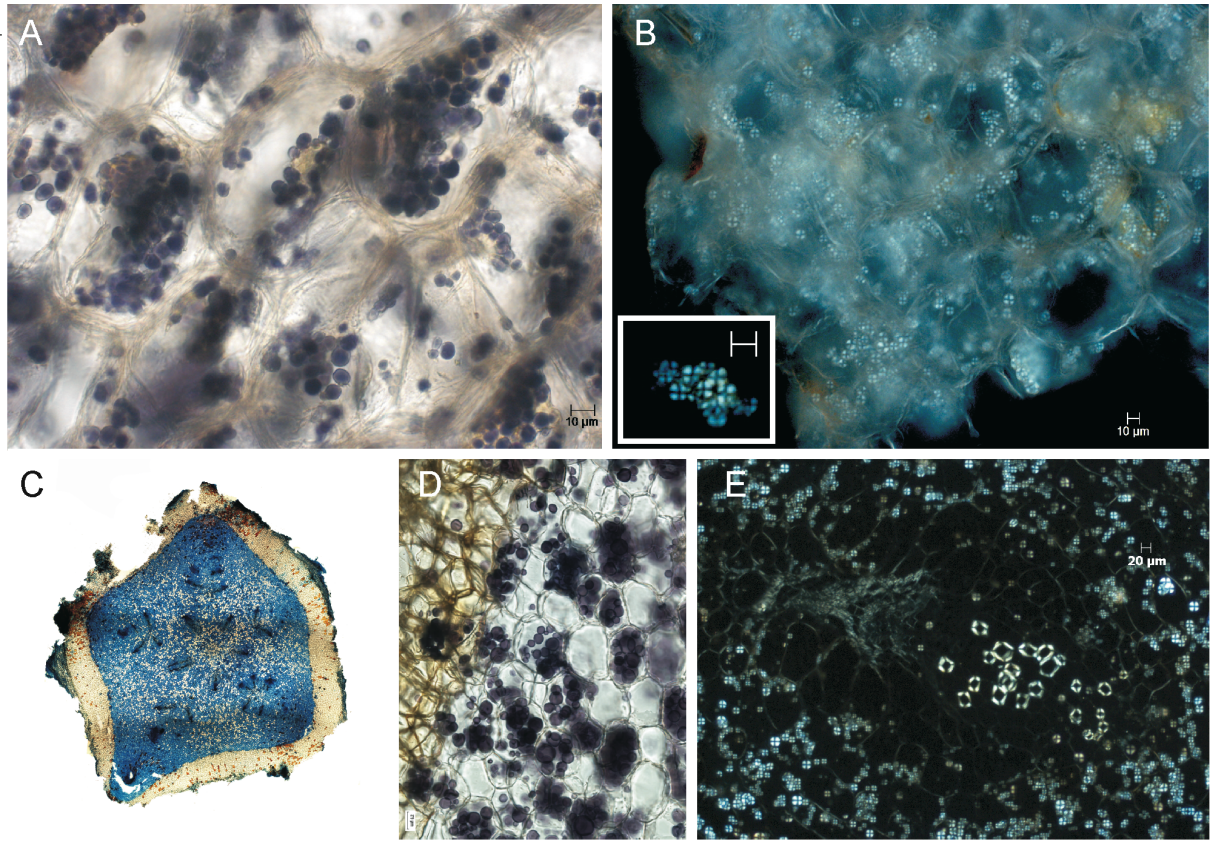






Figure 6

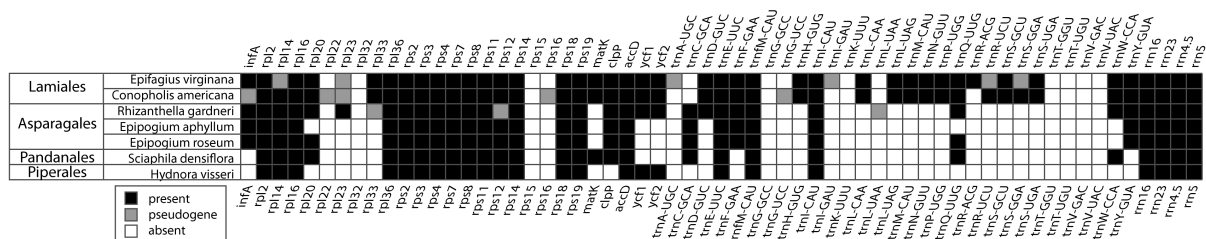


Figure 7

